A rapid and convenient method for detecting a broad spectrum of malignant cells from malignant pleuroperitoneal effusion of patients using a multifunctional NIR heptamethine dye

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Detection of malignant cells from malignant effusion is crucial to establish or adjust therapies of patients with cancer. The conventional qualitative detection in malignant pleuroperitoneal effusion is cytological analysis, which is time-consuming and complicated. Therefore, a faster and more convenient detection strategy is urgently needed. In this study, we report a rapid method to detect malignant cells from malignant pleuroperitoneal effusion (hydrothorax and ascites) of patients using IR-808, a tumor-targeted near-infrared (NIR) fluorescent heptamethine dye (tNRI dye), which exhibited superior labeling efficacy without specific conjugation to biomarkers. The targeted imaging performance toward malignant cells using IR-808 was confirmed by comparing with normal cells, and the fluorescence stability assay of IR-808 in malignant effusion was performed from 1 h to 48 h. In order to save time and dose, the incubation time and concentration were optimized to 10 min and 5 μM, which were used to detect malignant cells from 28 clinical samples of malignant pleuroperitoneal effusion. The results revealed that IR-808 could be internalized selectively by malignant cells of samples, and these malignant cells could be easily distinguished from normal cells under a fluorescence microscope. The positive rates between cytological analysis and the IR-808 staining method were 86% (24/28) and 79% (22/28), respectively. An excellent concordance level (Kappa = 0.752, $P < 0.001$) was observed between the two methods. Our results indicated that IR-808, a new NIR fluorescent heptamethine dye with unique optical imaging and tumor targeting properties, could provide a fast and simple way to detect a broad spectrum of malignant cells from malignant pleuroperitoneal effusion in patients.

Introduction

Patients with cancer often have abnormal fluid accumulation relevant to the imbalance rate between the entry and exit of fluids into/from the pleuroperitoneal space.[1–3] Malignant pleuroperitoneal effusion (malignant hydrothorax and ascites) is mostly caused by the invasion and metastasis of malignant cells in pleuroperitoneal membranes, which is common in patients in the advanced stage and related to a poor prognosis with a short median survival of about 9–17 months after first-diagnosis.[4] Since the presentation of malignant pleuroperitoneal effusion precludes patients from curative resection, the detection of malignant pleuroperitoneal effusion has great influence on the clinical decision.

In clinical practice, the malignant pleuroperitoneal effusion is determined based on the detection of malignant cells from effusion by cytological analysis.[5–8] Cytological features of malignant cells are characterized by the presentation of nuclear atypia, multinucleation, inclusion body and cell cluster formation.[9,10] However, the similarity of mesothelial cells to malignant cells in morphology often makes the distinction difficult, especially when the number of cells in effusion is small.[11–13] Furthermore, the procedure of cytological analysis is complicated, and its result is highly dependent on the operator's experience, which makes this method time-consuming and vulnerable to subjective bias.[14] Therefore, a faster and much simpler method to detect malignant cells from pleuroperitoneal effusion is urgently needed.

A new class of tumor-targeted near-infrared (NIR) fluorescent heptamethine dyes (tNRI dyes) has attracted great interest in

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biodetection and biomedical imaging.[15,16] These tNRI dyes may preferentially accumulate in the mitochondria of malignant cells, but not in normal, dead or apoptotic cells.[17] And the NIR emission (700–900 nm) of these dyes significantly increases the sensitivity of detection due to the low background from the autofluorescence of effusion in this wavelength range. Typically, the contrast index value of cells stained by tNRI dyes can exceed 20, while a contrast index more than 2.5 is regarded as substantial accumulation in malignant cells.[18,19] In addition, the fluorescence of tNIR dyes remains stable during the preparation of effusion samples, indicating that these heptamethine dyes may be promising candidates for malignant cell targeting and detection.[20–25]

Recently, Yang *et al.* found that malignant cells could be tested using tNIR dye IR-783 after mixing human prostate malignant cells with human blood cells. This dye was sufficiently sensitive to detect as few as 10 malignant cells per milliliter in whole blood, indicating that this group of dyes has the potential to detect malignant cells in circulation.[26] However, to the best of our knowledge, the fluorescence detection ability of tNRI dyes has not been investigated in malignant pleuroperitoneal effusion. Therefore, we herein exploit the ability of tNRI dyes to detect malignant cells from clinical malignant effusion as a fast and simple complementary tool to cytological analysis.

In this research, a new tNIR dye with 808 nm emission (IR-808) was chosen to detect malignant cells from cultured cell lines and 28 clinical malignant pleuroperitoneal effusion samples. A conventional NIR dye Cy7 was taken as control to compare the imaging performance. And the positive rates and concordance level between cytological analysis and the IR-808 staining method were studied to compare the sensitivity to malignant cells in clinical samples.

Experimental section

Heptamethine dye and samples

The heptamethine dye IR-808 was kindly provided by Dr Chunmeng Shi from the Third Military Medical University at Chongqing of China, which was dissolved easily in water at 10 mM and stored at −20 °C before use. All the samples of the malignant pleuroperitoneal effusion in this study were collected from the Pathology Department of Jinling Hospital. The specimens examined in our study were also diagnosed pathologically.

Cell lines and cell culture

All human malignant cell lines were obtained from American Type Culture Collection (ATCC) and were maintained in ATCC recommended media supplemented with penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and 10% fetal bovine serum (GIBCO, USA) at 37 °C with 5% CO_2, including lung cancer (NCI-H460, A549), breast cancer (MDA-MB-231, MCF-7), colon cancer (HCT-116), glioma cancer (U87), renal cancer (786-O), cervical cancer (Hela), osteosarcoma (U-2OS), and human normal cells including embryonic kidney cells (293T), vascular endothelial cells (VE), embryonic lung fibroblast (HELF), and umbilical vein endothelial cells (HUVEC) were used as control.

Uptake and accumulation of IR-808 in normal and malignant cells *in vitro*

All cells were cultured at a density of 2×10^6 in a 35 mm Petri dish with cover slips, and incubated at 37 °C with 5% CO_2 for 24 h. The cover slips were incubated with IR-808 at a concentration of 20 μM for 20 min at 37 °C after the cells had reached a confluence of 70%. Then the nuclei of cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) after washing with phosphate-buffered saline solution (PBS) according to the kit instructions and fixed with 4% formaldehyde at 4 °C after washing again with PBS. After that, the cells were embedded with a cover slip in mounting medium for fluorescent imaging. Images were recorded by using a fluorescence microscope (Olympus IX71, Japan) equipped with fluorescent filter sets (excitation, 684–719 nm; emission, 740–950 nm).

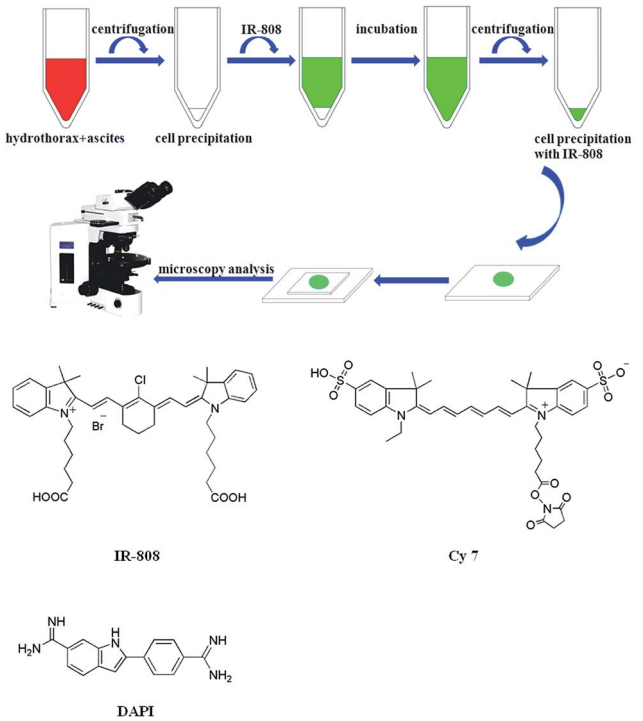
To further optimize the dose and incubation time, a group of 2×10^6 MCF-7 cells was chosen to incubate with IR-808 at the final concentrations of 1.25, 2.5, 5, 10, 20 μM for 20 min, and another group of MCF-7 cells was incubated for different times (5 min, 10 min, 30 min, 1 h, 8 h, and 24 h), then all cells were stained with DAPI, 4% formaldehyde in turn, and imaged with the fluorescence microscope at the same exposure time. The mean fluorescence intensities of the groups were statistically analyzed. Then MCF-7 cells were chosen to co-incubate with IR-808 and DAPI at the best concentration and time, the images were obtained subsequently.

Fluorescence stability assay of IR-808 in malignant pleuroperitoneal effusion

The fluorescence stability assay of IR-808 in ultra-pure water, and a malignant pleuroperitoneal effusion cell precipitation mixture with a concentration of 5 μM was performed at 37 °C for 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h using an IVIS Lumina XR (Xenogen Corporation-Caliper, Alameda, CA, USA) with NIR wavelength (745 nm excitation and 830 nm emission). Eventually the cell precipitates after centrifugation were added dropwise onto microscope slides and imaged by the same NIR imaging method.

Assessment of IR-808 in the malignant pleuroperitoneal effusion samples

All the samples of the malignant pleuroperitoneal effusion were collected after centrifugation at 1000 rpm for 5 min, leaving cell precipitates only. The cells were co-incubated with IR-808 and DAPI at a concentration of 5 μM for 10 min, and incubated with formaldehyde for 20 min, then smeared on microscope slides and observed according to the procedures described above. Malignant hydrothorax samples were chosen to incubate with IR-808 and cyanine dye Cy7, another common NIR heptamethine cyanine, in order to compare the imaging performance toward malignant cells. Images and cell count were recorded by using a special NIR fluorescence microscope. The processes were consistent with that shown above. The whole diagnosis



Scheme 1 A schematic representation showing the whole process of diagnosing malignant cells from malignant pleuroperitoneal effusion (hydrothorax and ascites) using fluorescent heptamethine dye IR-808, and the chemical structures of IR-808, Cy7 and DAPI.

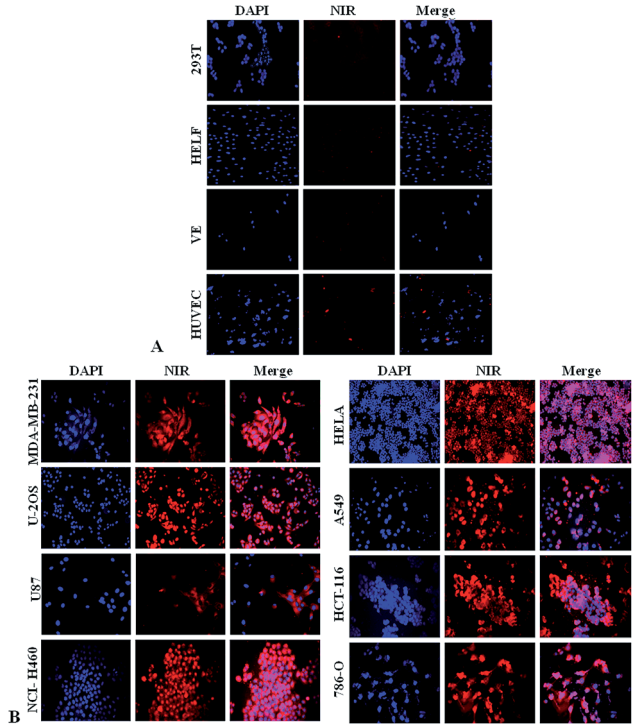


Fig. 1 (A) Normal human cells (293T, HELF, VE, and HUVEC) showed no uptake of IR-808 after incubation. (B) Human malignant cell lines (MDA-MB-231, U-2OS, U87, NCI-H460, Hela, A549, HCT-116, and 786-O) showed significant uptake of IR-808 under similar staining and imaging conditions. Results are shown with images obtained from the nuclei of cells stained with 4′,6-diamidino-2-phenylindole (DAPI), IR-808 staining (NIR), and by merging of the two images (Merge). All images were acquired at 100× magnification.

process is shown briefly in Scheme 1 with the chemical structures of IR-808, Cy7 and DAPI.

Histological and statistical analyses

The collected cells of malignant pleuroperitoneal effusion were smeared on microscope slides after centrifugation at 1000 rpm for 5 min, then fixed in 95% ethanol for 30 min, and stained with hematoxylin and eosin for 2 min. The whole slides needed washing for 1 h and drying for pathological analysis.

All statistical analyses were performed using the SPSS 19.0 software program (SPSS, Chicago, IL). The statistical significance of the two detection methods was evaluated *via* the McNemar paired chi-square test and the Kappa test from descriptive statistics. Data were expressed as mean ± SD, a two-sided P value of less than 0.05 was considered statistically significant in all the statistical tests.

Results and discussion

Preferential accumulation of IR-808 in malignant cells

Cells labelled with IR-808 were observed using a fluorescence microscope with special near infrared absorption and emission spectra. The uptake and accumulation of IR-808 were studied in four human normal cell lines (Fig. 1A) and eight human malignant cell lines (Fig. 1B). In this experiment, we used the compatible incubation time of 20 min and the concentration of 20 μM. The dye was not found to accumulate in human normal embryonic kidney cells (293T), vascular endothelial cells (VE), embryonic lung fibroblasts (HELF), and umbilical vein endothelial cells (HUVEC), but in human lung cancer (NCI-H460, A549), breast cancer (MDA-MB-231), colon cancer (HCT-116), glioma cancer (U87), renal cancer (786-O), cervical cancer (Hela), and osteosarcoma (U-2OS) cells. The difference in IR-808 staining among all cell lines are shown in Fig. 1. It was clear that only malignant cells could be stained, while normal cells were unstained. There were no apparent differences among all these malignant cells, indicating that this dye, preferentially accumulated in malignant cells, was not affected by different cell surface receptors or biological factors, which further confirmed that the IR-808 staining method was a broad spectrum technology for detecting malignant cells.

The targeted imaging properties of the lipophilic dye were attributed to the higher negative transmembrane potentials inside the mitochondria of malignant cells than that of normal cells, verified by co-localization with a membrane potential-dependent mitochondrial tracker, rhodamine 123, which made IR-808 a broad spectrum detection agent because most malignant cells have been identified with higher mitochondrial membrane potentials.[27–30] In addition, viable malignant cells could be distinguished from dead cells or apoptotic cells because the whole reaction pathway was energy-dependent.

A few stains in HUVEC cell lines were observed by NIR fluorescence imaging, because a few non-specific stains or spots appeared inevitably on the slides.

Effect of IR-808 on concentration and time tests

In order to determine whether IR-808 with a low concentration had good imaging ability, we chose a gradient ranging from 20 to 1.25 μM to dye the human breast cancer MCF-7, and other experimental conditions such as the cell count, the treatment temperature and the exposure times were consistent for each group. As shown in Fig. 2A, imaging differences were detected as the change of concentration in these assays, and malignant cells could be dyed even at a concentration of 1.25 μM. The concentration of 5 μM was chosen for the next study because the NIR fluorescence signal at this concentration could reach a contrast value over 20. So a low dose of IR-808 could be chosen for incubation, and the optical properties of targeted imaging were not apparently affected, so this new method may be more cost-saving than clinical practice.

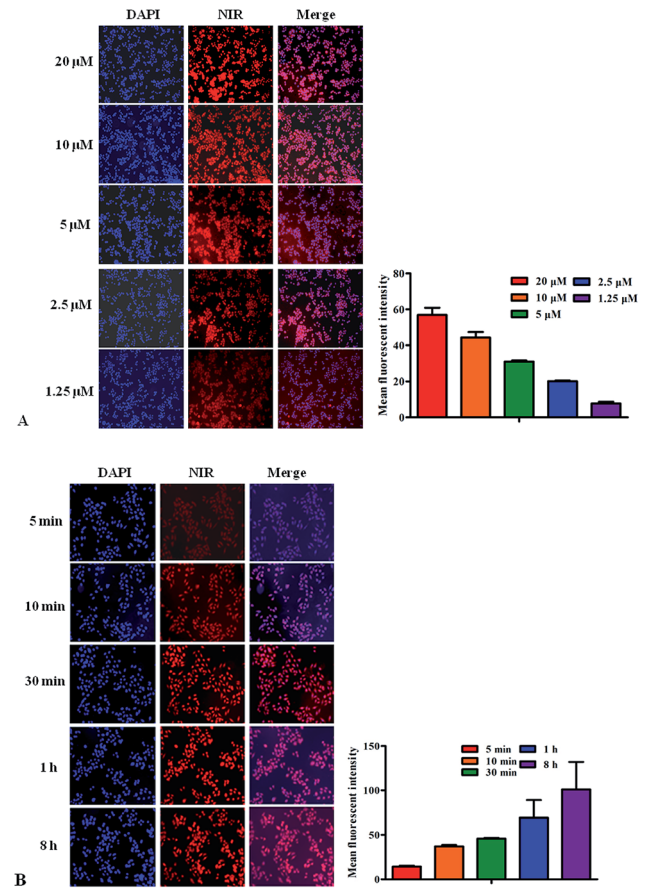


Fig. 2 To further optimize the dose and time, 2×10^6 MCF-7 cells were chosen to incubate with IR-808 at the final concentrations of 1.25, 2.5, 5, 10, and 20 μM for 20 min (A), and time points of 5 min, 10 min, 30 min, 1 h, and 8 h at 5 μM (B). The images obtained by staining with DAPI, IR-808 (NIR), and merging of the two images (Merge) are shown. The mean fluorescence intensities under different conditions were statistically analyzed. All images were acquired at 100× magnification.

From Fig. 2B, the targeted imaging was differentiated due to the incubation times. It was appropriate and enough to detect malignant cells when the cells were incubated with IR-808 for about 10 min, which was less than clinical routine operation. Further performance of co-incubation with DAPI and IR-808 was tested at 5 μM for about 10 min, compared with individual incubation. The results revealed that the detection performance was not influenced when samples were co-incubated with IR-808 and DAPI, indicating that the new method was timesaving without losing its accuracy (Fig. 3).

Optical properties of IR-808 in malignant pleuroperitoneal effusion samples

Zhang *et al.* demonstrated that the fluorescence signal of these dyes was pretty stable after formalin fixation, raising the possibility of developing sensitive means of detecting target sites especially for tumor evaluation.[31] As shown in Fig. 4A, the fluorescence intensity of IR-808 was investigated from 1 h to 48 h using an IVIS Lumina XR (745 nm excitation, 830 nm emission), which maintained a well, stable fluorescence intensity in malignant pleuroperitoneal effusion (hydrothorax and ascites) at the initial and final test time points, indicating that the fluorescence intensity of IR-808 was very stable for further imaging application. The photoluminescence of IR-808 in malignant pleuroperitoneal effusion showed a higher value, indicating that some large molecular agents in malignant effusion such as albumin, enzymes and carcinoembryonic antigen may be nonspecifically labelled by excess dyes, but this phenomenon could not affect malignant cellular imaging under the microscope. After 48 h, cell precipitates in malignant pleuroperitoneal effusion were collected and imaged after centrifugation, leading to the result that only the samples including malignant cells showed targeted imaging (Fig. 4B).

Detection of malignant cells from malignant pleuroperitoneal effusion

Encouraged by the above results, we then studied whether the dye could detect malignant cells from malignant pleuroperitoneal effusion of patients with lung cancer, ovarian cancer

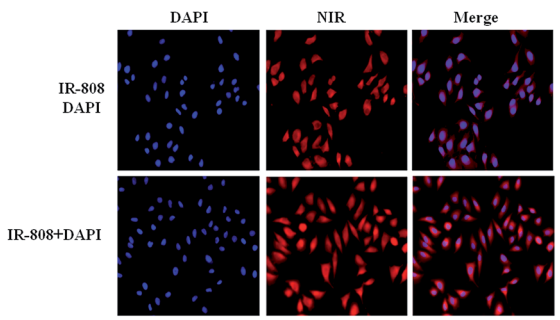


Fig. 3 To further test the effect of co-incubation with DAPI and IR-808 (IR-808 + DAPI), MCF-7 cells were chosen to incubate at the concentrations of 5 μM for about 10 min, compared with individual incubation (IR-808, DAPI). All images were acquired at 100× magnification.

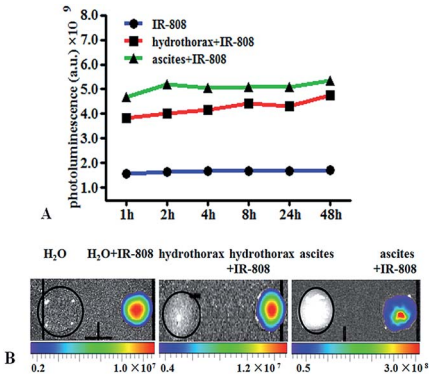


Fig. 4 The fluorescence intensity of malignant pleuroperitoneal effusion (hydrothorax and ascites) with IR-808 at different time points (1 h, 2 h, 4 h, 8 h, 24 h, and 48 h) was calculated (A). The cell precipitates from malignant pleuroperitoneal effusion were collected and imaged (B).

and breast cancer. Fig. 5 shows that malignant cells from malignant effusion samples could be stained after incubation with IR-808, while other cell types of the samples such as macrophages and mesothelial cells were not stained. The percentage of the stained malignant cells from the two samples was counted, respectively (76.42 ± 13.13; 32.48 ± 3.07), indicating that not all cell types could be visualized except malignant cells. Cytological analysis of malignant pleuroperitoneal effusion was conducted in parallel. Prominently, the whole procedure of the new detecting method costs about 1.5 hours

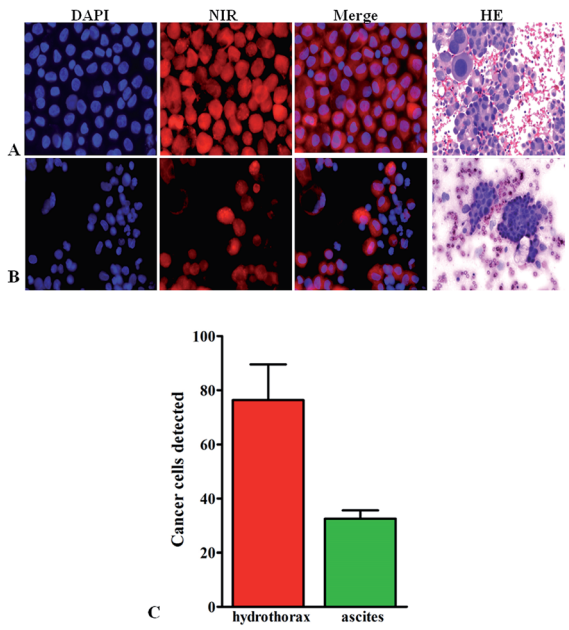


Fig. 5 Malignant cells of hydrothorax (A) and ascites (B) samples were analysed by DAPI, IR-808 staining (NIR), and merging of the two images (Merge). Malignant cells could be clearly observed after IR-808 NIR imaging, but other cell types of the samples were not stained. Cytological analysis (H&E) of malignant cells from malignant pleuroperitoneal effusion was conducted accordingly. All images were acquired at 400× magnification. The percentage of stained malignant cells in all cell types from the two samples was counted (C).

including centrifugation, washing, incubation, smearing and imaging, and the diagnostic report for cancerous or non-cancerous effusion could be obtained preliminarily by the detection of labelled cells. While cytological analysis needed much more time to wash and dry. So, the timesaving and convenient operations of the new detection method were more worthy than cytological analysis. But it could not be used to confirm the characteristics of malignant cells morphologically.

We also used Cy7 as control, it was found that no Cy7 retained in malignant cells in hydrothorax samples (Fig. 6), suggesting that not all fluorescent heptamethine dyes just like Cy7 were attached to the malignant cells typically in a broad spectrum range and characterized with simultaneous tumor targeting and NIR imaging capabilities.

Comparison of detection rate between cytological analysis and the new staining method

Twenty eight samples (17 hydrothorax and 11 ascites) in this study were collected from definite diagnostic patients (16 males and 12 females; age: 61.44 ± 10.26) from May to September in 2013 (Table 1). Using cytological analysis and the IR-808 staining method, the positive rates in the 28 samples with cancer were 86% (24/28) and 79% (22/28). There was a good concordance level (Kappa = 0.752, $P < 0.001$) between these two methods. There were two samples detected by the cytological technique but not by the IR-808 staining method. The reason may include (1) the quenching of NIR fluorescence occurred with some inevitable factors; (2) the integrities of some cells were destroyed; (3) the exposure time of the specimens was too long and thus resulted in photobleaching. All IR-808 positively stained samples were confirmed by cytological analysis, indicating its high sensitivity. There were four samples unidentified by these two methods owing to the reason that the malignant pleuroperitoneal effusion may be caused by non-cancerous lesions, such as the imbalance between osmotic pressure and blood pressure, or the stimulation of inflammation in patients with cancer.[32] It is necessary to combine the two methods to meet the diagnosis needs.

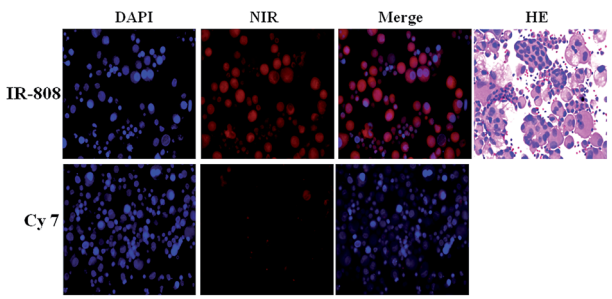


Fig. 6 Malignant cells in hydrothorax samples were dyed with IR-808 and Cy7. DAPI, IR-808 or Cy7 staining (NIR), merging of the two images (Merge) and H&E were conducted in turn. Malignant cells could be clearly observed after IR-808 NIR imaging, but Cy7 could not. All images were acquired at 400× magnification.

Table 1 Comparison of the results of the two methods used in malignant cell detection in 28 patients

	Case No.	Hydrothorax	Ascites	
Overall	28	17	11	
Sex				
Male	16	12	4	
Female	12	5	7	
Age (years)				
≤55	5	3	2	
>55	23	19	4	
Cytological analysis	24	17	7	Kappa = 0.752
IR-808 staining	22	15	7	$P < 0.001$

Conclusions

At present, the clinical qualitative means of detection for malignant cells in malignant pleuroperitoneal effusion is dependent on routine cytological analysis. However, the operational steps of cytological analysis are complicated and time-consuming. In this study, our fast and convenient diagnosis strategy only needs centrifugation, incubation and washing, but has high accuracy and sensitivity to diagnose malignant cells in malignant pleuroperitoneal effusion. The key to our strategy is the use of multifunctional NIR heptamethine dye IR-808, which shows the malignant cell targeting properties of malignant effusion. To the best of our knowledge, this is the first report of IR-808 as a NIR optical imaging agent to label malignant cells in clinical specimens, and this method could provide information for cytological analysis. The present results suggest that the IR-808 staining method is sensitive, convenient, low-cost and timesaving; it may be useful as a tool for cytological analysis and other clinical applications in future.

Acknowledgements

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